卡博替尼联合 PD-L1 单抗对黑色素瘤小鼠移植瘤的抑制作用机制

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摘　要：【目的】探讨卡博替尼联合 PD-L1 单抗对恶性黑色素瘤小鼠皮下移植瘤生长的影响及作用机制。【方法】利用体外培养的小鼠黑色素瘤细胞 B16-F10 建立小鼠皮下移植瘤模型，成瘤后将小鼠随机分为盐水对照组、溶剂对照组、PD-L1 单抗组、卡博替尼组和卡博替尼联合 PD-L1 单抗组（联合组）。观察肿瘤生长情况及每 2 d 测量一次肿瘤体积。肿瘤体积达到 2 000 mm^3 或是组间差距有显著统计学差异时定义为研究终点。达到研究终点后处死小鼠取材，流式细胞学检测肿瘤组织中浸润免疫细胞情况，包括 $CD4^+$、$CD8^+$T 淋巴细胞及髓源性抑制细胞（MDSC）。另将体外培养的 B16-F10 细胞分别经不同药物处理后，行流式细胞术检测细胞凋亡情况；Western blot 检测 AKT、p-AKT、mTOR 和 p-mTOR 蛋白表达水平。【结果】B16-F10 黑色素瘤移植瘤模型显示，PD-L1 单抗组没有明显抑瘤作用，卡博替尼组与联合组均有明显抑瘤效应，联合组抑瘤效果较卡博替尼组更为显著，差异具有统计学意义（P=0.001 5）。在体外实验中，药物处理 24 及 48 h 后流式细胞术检测 B16-F10 细胞凋亡，结果显示联合组与卡博替尼组相比，B16-F10 细胞凋亡比例均显著增高，差异具有统计学意义（24 h：P=0.003 5；48 h：P=0.002 9）。Western blot 检测显示联合组及卡博替尼组对 AKT 和 mTOR 蛋白水平无明显影响，但均可下调 p-AKT 和 p-mTOR 蛋白水平，联合组作用更为显著。【结论】卡博替尼联合 PD-L1 单抗有协同抗肿瘤作用，这种协同抗肿瘤作用机制可能是通过促进 B16-F10 细胞凋亡及抑制 AKT/mTOR 通路所致。

关键词：黑色素瘤；卡博替尼；PD-L1 单抗
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Cabozantinib Combined with Anti-PD-L1 Antibody Produced Antitumor Effect on Mice Melanoma Xenograft and its Mechanism

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Abstract:【Objective】The aim of this study was to investigate the effect and mechanism of cabozantinib combined with anti-PD-L1 antibody on the growth of subcutaneous transplanted malignant melanoma in mice.【Methods】Established mouse subcutaneous xenograft model using mouse melanoma cell line B16-F10, and then randomly divided into five groups: saline control group, vehicle control group, anti PD-L1 antibody group, cabozantinib group, cabozantinib in combined with anti-PD-L1 antibody group (combination group). Tumor growth was observed and tumor volume was measured every 2 days. The research endpoint was defined as when the tumor volume reached 2 000 mm^3 or the difference between the groups was statistically significant. Then the mice were sacrificed and tissue samples were taken at the endpoint of the study. Infiltrating immune cells including $CD4^+$, $CD8^+$ T lymphocytes and myelogenous suppressor cells (MDSC) were detected by flow cytometry. In addition, B16-F10 cells cultured in vitro were treated with different drugs, the apoptosis was detected by flow cytometry, and the protein expressions of AKT, p-AKT, mTOR and p-mTOR were

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detected by western blot assay.【Results】B16-F10 melanoma xenograft model showed that anti-PD-L1 antibody group had no obvious antitumor effect, while both cabozantinib group and combination group produced significant antitumor effect, and the combination group had more obvious antitumor effect compared to cabozantinib group (P=0.001 5). B16-F10 cells were treated with different drugs in vitro, and the apoptosis rate of the combination group was significantly higher than that of cabozantinib group at 24 h and 48 h, respectively (24 h: P=0.003 5; 48 h: P=0.002 9). Western blot assay showed that the combination group and cabozantinib group had no significant effect on the protein expression of AKT and mTOR, but both could reduce their phosphorylation levels, and the combination group was more remarkable.【Conclusion】Cabozantinib in combined with anti-PD-L1 antibody had synergistic anti-tumor effect, which might be achieved by promoting B16-F10 cells apoptosis and inhibiting of AKT/mTOR pathway.

Key words: melanoma; cabozantinib; anti PD-L1 antibody

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黑色素瘤是最具侵袭性的肿瘤之一，虽然仅占所有皮肤癌的5%，但约80%的皮肤癌死亡与黑色素瘤相关[1]。晚期黑色素瘤中位生存时间仅为8~10个月，5年生存率低于10%[2]。随着近年来免疫治疗的发展，免疫检查点抑制剂的应用彻底改变了黑色素瘤传统的治疗模式。免疫检查点抑制剂ipilimumab（阻断CTLA-4的抗体）及PD-1单抗（阻断PD-1的抗体）先后被FDA批准用于治疗不可手术切除的黑色素瘤病人。临床试验结果显示PD-1单抗在晚期黑色素瘤中客观有效率约31%~37%[3-5]，2年总生存率为55%[6]。另一个免疫检查点抑制剂PD-L1单抗atezolizumab在PD-L1表达5%以上的局部晚期或转移性尿路上皮癌患者中有效率达26%[7]。在ⅢB或Ⅳ期非小细胞肺癌治疗中，atezolizumab与多西他赛相比，总生存时间提高2.9个月[8]。临床试验结果显示，PD-L1单抗相比PD-1单抗及CTLA-4抑制剂，副作用更小，更适合与传统治疗方式联合使用。对于存在BRAF突变的晚期恶性黑色素瘤患者，BRAF抑制剂的应用使得患者在短期内受益显著，有效率高达48%~53%，但疗效持续时间短，容易出现耐药，中位无进展期＜6个月[9-10]。鉴于免疫检查点抑制剂起效慢（中位起效时间为8~12周）、有效率低、疗效持久，而小分子抑制剂起效时间快、近期有效率高的特点，一些研究者试图将两者联合，观察是否存在协同抗肿瘤效果。有研究报道在BRAF突变的恶性黑色素瘤模型中，BRAF抑制剂联合PD-1或PD-L1单抗产生明显协同抑瘤作用[11]。但由于BRAF突变患者仅占黑色素瘤病人40%~60%[12]，中国黑色素瘤患者BRAF突变率更低，约25.5%[13]。因此限制了它联合免疫检查点抑制剂的广泛应用。卡博替尼是一个多靶点小分子酪氨酸激酶抑制剂（主要包括MET、VEGFR1、VEGFR2、VEGFR3、ROS1、RET、AXL、NTRK、KIT 9个靶点）。由于其作用靶点丰富，且无基因突变要求，可能是一种更适合与免疫检查点抑制剂联合使用的小分子靶点药物。2017年发表在Nature上的一篇文章报道：在一种对于去势治疗耐药的前列腺癌小鼠模型上，卡博替尼联合抗PD-1抗体显示明显协同抑瘤效果[14]。基于PD-L1单抗、卡博替尼作用机制及先前研究成果，将进一步评估卡博替尼联合PD-L1单抗是否在黑色素瘤中同样有协同抑瘤效果及相关作用机制。

1　材料与方法

1.1　抗体和试剂

卡博替尼（XL184，BMS-907351）购于Selleck公司，抗鼠PD-L1单抗（BE0101-100MG）购于Bio X Cell公司。内参GAPDH抗体、AKT抗体、p-AKT抗体（磷酸化位点Ser473）、m-TOR抗体、p-mTOR抗体均购自Cell Signaling Technology公司，二抗抗兔抗体购于proteintech公司。Annexin V-FITC/PI试剂盒购于南京凯基生物有限公司（KGA108）。抗鼠CD4-APC、CD8-PE、Gr-1-PE、CD11b-FITC流式抗体均购于eBioscience公司。

1.2　细胞培养

黑色素瘤细胞株B16-F10由本实验室保存，细胞培养于含10%胎牛血清的DMEM培养液中（含100 U/mL青霉素、100 μg/mL链霉素），于37 ℃、体积分数为5%的CO_2孵箱中培养。

1.3 动物实验

无特殊病原菌(SPF)C57BL/6小鼠40只,6~8周龄,雄性,体质量18~20 g,购自中山大学实验动物中心东校区[生产许可证号为SCXK(粤)2016-0029],健康状况良好,进动物房前常规检疫一周,检疫后饲养于中山大学北校区动物实验中心[动物实验室许可证号SYXK(粤)2015-0102],室温为(23±2)℃,相对湿度为(55±5)%,每天光照、黑暗交替各12 h。自由进食、饮水。动物实验伦理批件号为:L102042017060C。

B16-F10细胞的培养条件如上所述。细胞培养至对数生长期,约长满至80%~90%时收取细胞并计数,使用无血清的DMEM将细胞浓度调整为2×10^6/mL,2×10^5/只,接种于小鼠右侧腋窝皮下成瘤。种瘤第3天,待肿瘤体积长至50~100 mm^3时,将40只小鼠随机分成5组:盐水组(200 μL/次,第0、3、6、9天,腹腔注射),溶剂组(100 μL/d,灌胃),卡博替尼组(15 mg/kg/d,灌胃),PD-L1单抗组(200 μg/只,第0、3、6、9天,腹腔注射),卡博替尼(15 mg/kg/d,灌胃)联合PD-L1单抗组(200 μg/只,第0、3、6、9、12、15天,腹腔注射;联合组)。溶剂组和PD-L1单抗组治疗持续时间为11 d,卡博替尼组和联合组治疗持续时间为21 d。每2天测1次小鼠体重及肿瘤体积。肿瘤体积(V) $=a\times b^2/2$(a为长径,b为短径)。达到研究终点时,进行颈椎脱臼处死,取瘤,测瘤重量,拍照及进行流式细胞学分析。

1.4 流式细胞术检测细胞表面分子

5组小鼠处死后分别取其肿瘤组织,从每个肿瘤中取适量肿瘤组织转移至EP管中,剪碎组织成泥状,加0.1 μg/L IV型胶原酶(Sigma,美国)在37℃摇床下孵育40 min,加入含体积分数1% FBS的PBS终止反应,2 000 r/min(r=16.8 cm),离心5 min,去上清,每管加入2 mL红细胞裂解液(Sigma,美国)混匀,冰上裂解5 min,2 000 r/min(r=16.8 cm),5 min,去上清,PBS清洗2次后,去上清,加入200 μL PBS重悬细胞,将其均匀分装至两个EP管中,1管加入APC偶联的抗小鼠CD4及PE偶联的抗小鼠CD8流式抗体冰上避光染色30 min,另一管加入FITC偶联的抗小鼠CD11b及PE偶联的抗小鼠Gr-1流式抗体冰上避光染色30 min,加入1 mL PBS终止染色,1 500 r/min(r=16.8 cm),5 min,去上清,300 μL PBS重悬,上机检测。流式分析软件采用FlowJo_V10。

1.5 流式细胞术检测凋亡率

B16-F10细胞接种于12孔板中,接种密度为1×10^5个细胞/孔组,加药处理24 h;接种密度为0.5×10^5个细胞/孔组,加药处理48 h,待细胞贴壁后给药处理:对照孔DMSO(1.1 g/L),卡博替尼孔(20 μmol/L),PD-L1单抗孔(50 ng/L),卡博替尼(20 μmol/L)联合PD-L1单抗(50 ng/L)孔,药物处理后分别在24 h和48 h终止反应并收集细胞样本,按照Annexin V-FITC/PI试剂盒实验步骤染色。通过流式细胞术(贝克曼流式细胞仪:Gallios荧光八色)测量Annexin V-FITC/PI荧光水平。Annexin V-FITC阳性、PI阴性或Annexin V-FITC、PI双阳性细胞被定义为凋亡细胞。

1.6 Western blot检测蛋白表达水平

B16-F10细胞接种于6孔板中,密度为2×10^5个细胞/孔,各孔分别予以DMSO(1.1 g/L,对照组),卡博替尼(20 μmol/L),PD-L1单抗(50 ng/L),卡博替尼(20 μmol/L)联合PD-L1单抗(50 ng/L,联合组)药物处理24 h。收集各实验组及对照组细胞,PBS清洗2次,加入RIPA及蛋白酶抑制剂冰上裂解细胞30 min,4 ℃下离心20 min(12 000 r/min,r=16.8 cm),收集上清,BCA法测量蛋白浓度。电泳时每孔上样量为20 μg。用十二烷基硫酸钠-聚丙烯酰胺凝胶电泳(SDS-PAGE)分离蛋白,低温条件下(冰浴)湿转电转至PVDF膜,体积分数为5%脱脂奶粉室温封闭1 h,TBST洗膜4次,分别加入用一抗稀释液的AKT(稀释度1∶1 000)、p-AKT(稀释度1∶1 000)、m-TOR(稀释度1∶1 000)、p-mTOR(稀释度1∶1 000)一抗4 ℃孵育过夜,TBST洗膜4次,分别用相应二抗室温孵育1 h,TBST洗膜4次后化学发光仪发光显影。

1.7 统计分析

应用Graphpad 7.0及SPSS 17.0软件进行绘图及统计分析。多组均数差异检验单因素时根据是否符合正态分布和方差齐性采用单因素方差分析(one-way ANOVA)或非参数检验(nonparametric tests),多重比较采用LSD-检验或Bonfferroni法。所有的统计学检验均采用双侧检验,$P<0.05$定义为有统计学差异,多重比较时根据两两比较次数调整检验水准α值,即$P<\alpha$定义为有统计学差异。

2　结　果

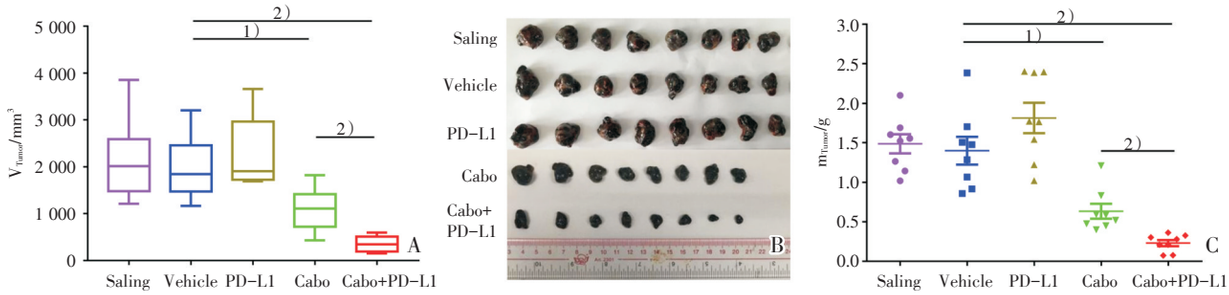
2.1　卡博替尼和PD-L1单抗对B16-F10黑色素瘤移植瘤的生长影响

种瘤第3天，将小鼠随机分为盐水对照组、溶剂对照组、PD-L1单抗组、卡博替尼组、卡博替尼联合PD-L1单抗组（联合组）5组，分别给予对应药物处理。结果显示，药物处理第0~4天，5组肿瘤体积差异不明显。药物处理第6~10天，盐水组，溶剂组和PD-L1单抗组的肿瘤体积迅速增长（在药物处理第10天，3组平均肿瘤体积接近2 000 mm^3，达到研究终点），而卡博替尼组和联合组的肿瘤生长较为缓慢。药物处理第0~20天过程中，小鼠肿瘤体积结果显示，PD-L1单抗组无明显抑瘤效果，卡博替尼单药与联合组均可明显抑制B16-F10移植瘤生长。与卡博替尼单药相比，联合组抑瘤效果更为明显，组间差异有统计学意义（P<0.001；图1A）。

到达研究终点时，五组肿瘤大小中联合组最小（图1B）。各组小鼠肿瘤质量结果显示盐水对照组、溶剂对照组、PD-L1单抗3组无明显统计学差异，而联合组、卡博替尼组与溶剂对照组相比均有统计学差异［联合组 *vs.* 溶剂对照组：（0.23±0.04）g *vs.*（1.40±0.18）g，P=0.001；卡博替尼组 *vs.* 溶剂对照组：（0.63±0.09）g *vs.*（1.40±0.18）g，P=0.002；图1C］。联合组肿瘤重量最轻，与卡博替尼组相比有统计学差异［联合组 *vs.* 卡博替尼组：（0.23±0.04）g *vs.*（0.63±0.09）g，P=0.001；图1C］。

2.2　流式细胞术检测肿瘤组织中$CD4^+$、$CD8^+$T淋巴细胞及MDSCs比例变化

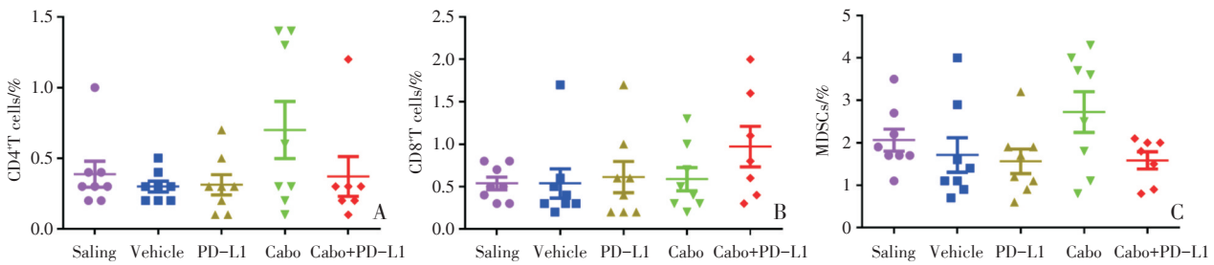
5组新鲜肿瘤组织制成单细胞悬液后，通过流式细胞学分析各组肿瘤组织中$CD4^+$和$CD8^+$T淋巴细胞及MDSC比例。结果显示盐水对照组、溶剂对照组、PD-L1单抗、卡博替尼组、联合组肿瘤中$CD4^+$T淋巴细胞比例（%）分别为0.38±0.09、0.30±0.04、0.31±0.07、0.70±0.20、0.37±0.14，各组之间无明显统计学差异（P=0.589）；$CD8^+$T淋巴细胞比例（%）分别为0.54±0.07、0.54±0.17、0.61±0.18、0.59±0.14、0.97±0.24，联合组较其他四组$CD8^+$T淋巴细胞比例有所增高，但无显著统计学差异（P=0.425）；MDSC比例（%）分别2.06±0.26、1.71±0.41、1.56±0.29、2.72±0.48、1.59±0.20，各组之间亦无明显统计学差异（P=0.258；图2）。



Cabozantinib（Cabo）in combined with anti-PD-L1 antibody（PD-L1）enhanced the efficacy of antitumor in vivo. A：Tumor volume in C57/B6 mice at the end of the study. Tumor diameters were measured every two days and tumor volume were calculated. Saline，Vehicle and PD-L1 group were administrated for 11 days（0-10 day），while Cabo and Cabo+PD-L1 groups administration time for 21 days（0-20 day）. Except Cabo+PD-L1 group for 8 mice，Saline，Vehicle，PD-L1 and Cabo for 9 mice per group. Testing difference of tumor volume between five groups used Nonparametric Tests（Kruskal-Wallis H，P<0.001）. Bonferroni method was used for multiple comparison. 1）P<0.01，2）P<0.001，adjusted test level for multiple comparisons α < 0.005 was statistically significant. B：C57/B6 mice were injected subcutaneously with 2×10^5 B16-F10 cells. Mice were treated with Saline or Vehicle or Cabo and or PD-L1 from the 3th day. At the 10th day after treatment，Saline and Vehicle group reached the study endpoint，tumors were extracted and photographed，while Cabo and Cabo in combined with PD-L1 group treatment lasting the 20th day，tumors were extracted and photographed. C：Tumor weight for 8 mice every group at the end of the study. Testing difference of tumor weight between five groups used Nonparametric Tests（Kruskal-Wallis H，P<0.001）. Bonferroni method was used for multiple comparison. 1）P<0.01，2）P<0.001，adjusted test level for multiple comparisons α < 0.005 was statistically significant.

图1　不同药物处理后肿瘤体积、肿瘤大小及肿瘤质量情况

Fig.1　Tumor volume，tumor size and tumor mass after treatment with different drugs



Cabozantinib (Cabo) in combined with anti-PD-L1 antibody (PD-L1) did not significantly increase the proportion of $CD4^+$ or $CD8^+$ T cells or attenuate MDSC frequency in the tumor microenvironment. A, B: Quantification of intratumoural $CD4^+$ or $CD8^+$ T cells frequency by indicated treatments by flow cytometry (n=8, 8, 8, 8 and 7, respectively). Testing difference of $CD4^+$ T cells frequency between 5 groups used Nonparametric test (Kruskal-Wallis H, P=0.589), Testing difference of CD8+ T cells frequency between 5 groups used nonparametric test (Kruskal-Wallis H, P=0.425). C: Quantification of intratumoural MDSC frequency by indicated treatments by flow cytometry (n=8, 8, 8, 8 and 7, respectively). Testing difference of MDSC frequency between 5 groups used nonparametric test (Kruskal-Wallis H, P=0.258).

图2 不同药物处理后肿瘤组织中$CD4^+$、$CD8^+$ T cells和MDSC比例变化

Fig.2 The ratio of $CD4^+$, $CD8^+$ T cells and MDSC in tumor tissues after treatment with different drugs

2.3 凋亡检测

采用AnnexinV-FITC/PI双染法检测不同药物处理后B16-F10细胞的凋亡率。药物处理后24 h,对照组、PD-L1单抗组、卡博替尼组、联合组凋亡率(%)分别为2.10±0.40、2.05±0.15、8.95±0.57、15.13±0.83;药物处理后48 h,对照组、PD-L1单抗组、卡博替尼组、联合组凋亡率(%)分别为2.22±0.42、2.29±0.37、17.82±0.67、28.9±0.87。从结果可以看出,与对照组相比,卡博替尼组和联合组药物处理24及48 h后均可明显促进B16-F10细胞凋亡,差异具有统计学意义(24 h:P<0.001,对照组 *vs.* 联合组:(2.10±0.40)% *vs.* (15.13±0.83)%,P<0.001;48 h:对照组 *vs.* 卡博替尼组:P<0.000 1,对照组 *vs.* 联合组:P<0.001),而PD-L1单抗组无明显促凋亡作用。和卡博替尼单药相比,联合组促凋亡作用更为明显,具有显著统计学差异(24 h:卡博替尼组 *vs.* 联合组:P<0.001;48 h:卡博替尼组 *vs.* 联合组:P < 0.001;图3)。

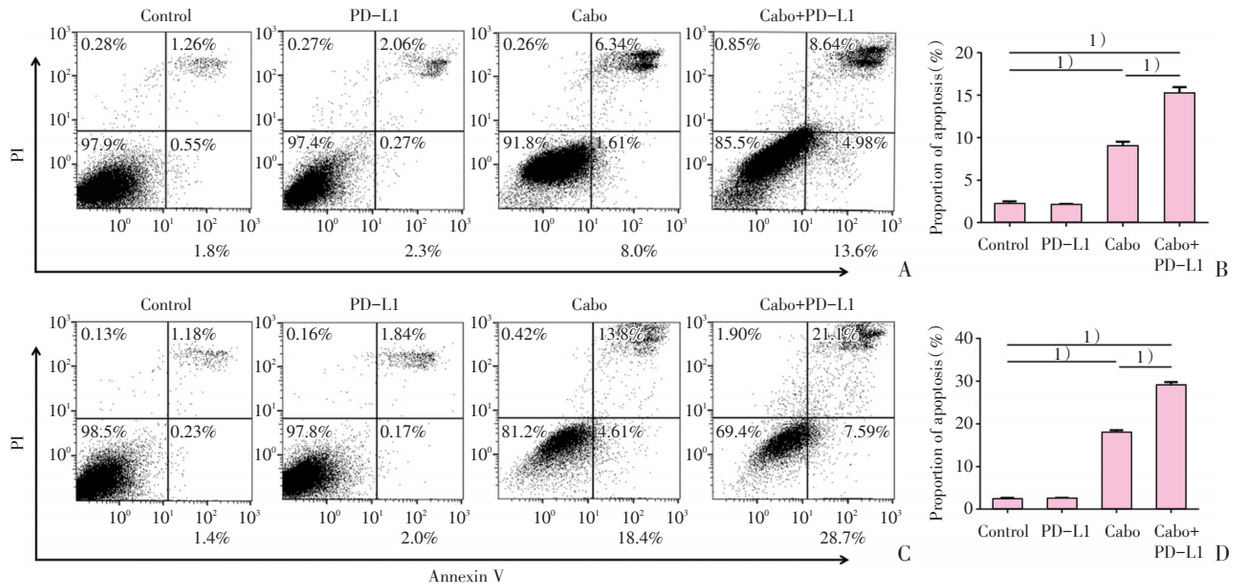
2.4 卡博替尼联合抗PD-L1抗体对AKT/mTOR通路的影响

体外实验中,药物处理后24 h收取蛋白进行western blot检测,结果显示四组药物处理后对AKT及mTOR总蛋白水平无明显影响,但卡博替尼组及联合组均明显下调p-AKT及p-mTOR蛋白水平,联合组较卡博替尼组更为显著(图4)。

3 讨 论

小分子靶向药物联合免疫检查点抑制剂在许多临床前研究中取得显著协同抑瘤作用。在恶性黑色素瘤中,有研究显示BRAF抑制剂联合PD-1或PD-L1单抗与BRAF抑制剂单药、PD-1或PD-L1单抗相比,$CD3^+$T细胞至少增加了7.5倍以上,且联合治疗产生明显协同抑瘤作用,显著延长移植瘤小鼠总生存时间[11]。在三阴乳腺癌中,小分子靶向药物MEK抑制剂联合免疫检查点抑制剂PD-1或PD-L1单抗,能明显上调TNBC细胞表面MHC及PD-L1表达,从而达到协同抗肿瘤效果。有研究报道在去势治疗耐药的前列腺癌的小鼠模型上,卡博替尼联合PD-L1单抗可协同下调肿瘤微环境中MDSC比例,减轻肿瘤微环境中免疫抑制因素,从而达到协同抗肿瘤作用[14]。除了临床前一些研究成果,目前有很多小分子靶向药物联合免疫检查点抑制剂的临床试验也正在进行中。

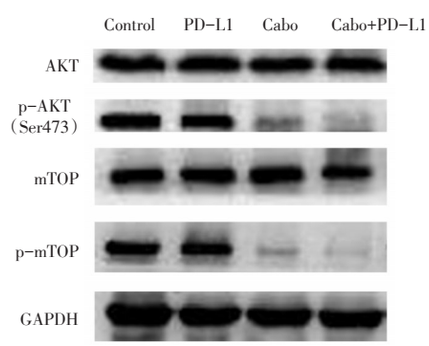
鉴于前人的研究结果:小分子靶向药物联合免疫检查点抑制剂大部分是通过调节肿瘤微环境中的免疫细胞而发挥协同抗肿瘤作用[11,14]。我们研究了多靶点药物卡博替尼联合PD-L1单抗在B16-F10黑色素瘤小鼠移植瘤模型中的抑瘤效果,并检测了肿瘤微环境中常见的免疫细胞。结果显示卡博替尼联合PD-L1单抗产生显著协同抑瘤效果(图1)。但是进一步通过流式细胞术分析肿瘤组织中浸润性免疫细胞发现,与其他四组相比,联合治疗组并没有募集更多的$CD4^+$T,$CD8^+$T淋巴细胞,也没有明显下调MDSC的比例。分析这一结果产生的原因可能是:卡博替尼是一个多靶点药物,其作用机制极其复杂,抗肿瘤途径繁



Cabozantinib (Cabo) in combined with anti-PD-L1 antibody (PD-L1) promoted the apoptosis of B16-F10 cells. A: Annexin V-PI assay of proportion of apoptosis of B16-F10 cell after treatment with Dmso (Control), PD-L1, Cabo or Cabo + PD-L1 for 24 h. Results from a representative experiment were shown in A. B: The number (%) below each flow cytometry panel indicated % of apoptosis cells (Annexin V positive and PI negative + Annexin V-PI double positive). Quantitative results of three separate experiments, as described in B. Each bar indicated mean±SEM. Testing difference of apoptosis rate between four groups used One-Way ANOVA (F=133, P<0.001). LSD-test was used for multiple comparison. 1)P<0.001, (One-Way ANOVA). C: Annexin V - PI assay of proportion of apoptosis of B16-F10 cell after treatment with DMSO (Control), PD-L1, Cabo or Cabo+PD-L1 for 48 h. Results from a representative experiment were shown in C. D: The number (%) below each flow cytometry panel indicated % of apoptosis cells (Annexin V positive and PI negative + Annexin V-PI double positive). Quantitative results of three separate experiments, as described in D. Each bar indicated mean±SEM. Testing difference of apoptosis rate between four groups used One-Way ANOVA (F=441, P<0.001). LSD-test was used for multiple comparison, 1)P<0.001.

图3　流式细胞术分析不同药物处理后B16-F10细胞的凋亡情况

Fig.3　Apoptosis of B16-F10 cells treated with different drugs was analyzed by flow cytometry



Western blot assay showed that both Cabozantinib (Cabo) and Cabo in combined with anti-PD-L1 antibody (PD-L1) could reduce the levels of p-AKT and p-mTOR protein, Cabo in combined with PD-L1was more remarkable.

图4　不同药物处理后p-AKT和p-mTOR的表达水平

Fig.4　Expression levels of p-AKT and p-mTOR after treatment with different drugs

多，与PD-L1单抗联合使用后，可能会从多个方面发挥协同抗肿瘤作用。而我们在体内实验中只是检测了肿瘤微环境中的常见免疫细胞这一种可能的机制。结果显示各组免疫细胞未见明显变化，因此不能通过免疫调节这一途径解释协同抗肿瘤的机制。可能存在其他协同抗肿瘤机制需要我们进一步研究。

由于体内实验的结果显示联合治疗组产生协同抗肿瘤的机制可能不是通过调节肿瘤微环境中的免疫细胞实现的，故在体外实验中，我们转变了研究思路。体外实验中，我们检测了不同药物处理后B16-F10细胞凋亡情况。药物处理24或48 h后，通过流式细胞术检测细胞凋亡，结果显示联合治疗组显著促进B16-F10细胞凋亡，和其他3组相比，差异具有显著统计学差异（图3）。从而我们猜测卡博替尼联合PD-L1单抗产生协同抗肿瘤的其中机制之一可能是通过促进B16-F10细胞凋亡实现的。

卡博替尼是一个多靶点药物(主要包括MET、VEGFR1、VEGFR2、VEGFR3、ROS1、RET、AXL、NTRK、KIT 9个靶点)。有文献报道卡博替尼的MET、RET及AXL靶点下游可以明显抑制AKT/mTOR通路,从而抑制肿瘤的生长[5]。也有研究报道AKT/mTOR通路可以调节PD-L1的表达[6]。鉴于此,我们用western blot法检测了不同药物处理后,AKT、p-AKT、mTOR、p-mTOR及PD-L1蛋白水平表达情况,结果显示卡博替尼及联合组均可下调p-AKT及p-mTOR蛋白水平,联合组尤为显著(图4)。但4组中PD-L1蛋白表达水平无明显变化(未放入文章中)。这个结果提示卡博替尼联合PD-L1单抗可显著抑制AKT/mTOR通路,但下游未能进一步调节PD-L1蛋白表达,推测可能是调节了AKT/mTOR下游的其他分子,需要进一步研究探索。

参考文献:

[1] Diamantopoulos P, Gogas H. Melanoma immunotherapy dominates the field[J]. Ann Transl Med, 2016, 4(14): 269.

[2] Garbe C, Eigentler TK, Keilholz U, et al. Systematic review of medical treatment in melanoma: current status and future prospects[J]. Oncologist, 2011, 16(1): 5-24.

[3] Topalian SL, Sznol M, McDermott DF, et al. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab[J]. J Clin Oncol, 2014, 32(10): 1020-1030.

[4] Robert C, Schachter J, Long GV, et al. Pembrolizumab versus Ipilimumab in advanced melanoma[J]. N Engl J Med, 2015, 372(26): 2521-2532.

[5] Hamid O, Robert C, Daud A, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma[J]. N Engl J Med, 2013, 369(2): 134-144.

[6] Schachter J, Ribas A, Long GV, et al. Pembrolizumab versus ipilimumab for advanced melanoma: final overall survival results of a multicentre, randomised, open-label phase 3 study(KEYNOTE-006)[J]. The Lancet, 2017, 390(10105): 1853-1862.

[7] Rosenberg JE, Hoffman-Censits J, Powles T, et al. Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial[J]. The Lancet, 2016, 387(10031): 1909-1920.

[8] Fehrenbacher L, Spira A, Ballinger M, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial[J]. The Lancet, 2016, 387(10030): 1837-1846.

[9] Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation[J]. N Engl J Med, 2011, 364(26): 2507-2516.

[10] Sosman JA, Kim KB, Schuchter L, et al. Survival in BRAF V600-mutant advanced melanoma treated with vemurafenib[J]. N Engl J Med, 2012, 366(8): 707-714.

[11] Cooper ZA, Juneja VR, Sage PT, et al. Response to BRAF inhibition in melanoma is enhanced when combined with immune checkpoint blockade[J]. Cancer Immunol Res, 2014, 2(7): 643-654.

[12] Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer[J]. Nature, 2002, 417(6892): 949-954.

[13] Si L, Kong Y, Xu X, et al. Prevalence of BRAF V600E mutation in Chinese melanoma patients: large scale analysis of BRAF and NRAS mutations in a 432-case cohort[J]. Eur J Cancer, 2012, 48(1): 94-100.

[14] Lu X, Horner JW, Paul E, et al. Effective combinatorial immunotherapy for castration-resistant prostate cancer[J]. Nature, 2017, 543(7647): 728-732.

[15] Song E-K, Tai WM, Messersmith WA, et al. Potent antitumor activity of cabozantinib, a c-MET and VEGFR2 inhibitor, in a colorectal cancer patient-derived tumor explant model[J]. Int J Cancer, 2015, 136(8): 1967-1975.

[16] Mittendorf EA, Philips AV, Meric-Bernstam F, et al. PD-L1 expression in triple-negative breast cancer[J]. Cancer Immunol Res, 2014, 2(4): 361-370.

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